The roles of Rirol and Ngrol genes in hairy root induction in Nicotiana debneyi

Seishiro Aoki a,*, Kunihiko Syōno b

a Department of General Systems Studies, Graduate School of Arts and Sciences, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo, 153-8902 Japan
b Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo, 112-8681 Japan

Received 14 December 1999; received in revised form 1 June 2000; accepted 7 June 2000

Abstract

The function of Rirol genes in TL-DNA of the Ri plasmid of Agrobacterium rhizogenes has been previously studied in Nicotiana tabacum and Daucus carota, but it was reported that these plants have a TL-DNA-similar sequence in their genome. We investigated the function of Rirol genes in N. debneyi by infection with A. tumefaciens harboring these genes, because the genome of N. debneyi does not contain a TL-DNA-similar sequence. The single gene RirolB induced adventitious roots in N. debneyi. Introduction of a DNA fragment that contained RirolB, RirolC, RiORF13 and RiORF14 resulted in more intense and earlier root formation than that of RirolB. Ngrol genes (NgrolB, NgrolC, NgORF13, and NgORF14) in the genome of Nicotiana glauca that are similar in sequence to Rirol genes were also examined. In contrast with Rirol genes, Ngrol genes did not induce adventitious roots on leaf segments of N. debneyi. Further infection analysis revealed that one of the reasons for this diversity of their functions might be the difference in the rolB region between the sequence of bacteria and plants. The difference in function between the genes of plants and bacteria is analyzed and the molecular evolution of Ngrol genes is discussed. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Agrobacterium rhizogenes; Hairy root; Ngrol genes; Nicotiana debneyi; Nicotiana glauca; rol genes

1. Introduction

Agrobacterium rhizogenes is a soil bacterium that is involved in the pathogenesis of hairy root disease in many dicotyledonous plants. This pathogenesis is caused by the transfer of one or two DNA fragments (T-DNA) from the Ri plasmid of bacteria to the plant genome. The agropine-type strain of A. rhizogenes has two T-DNA, TL-DNA and TR-DNA [1,2]. TL-DNA is believed to play the lead role in hairy root disease because cucumopine- and mannopine-type strains contain a single T-DNA that is homologous to the TL-DNA sequence [3,4]. Analysis by transformation with T-DNA genes has shown that rol genes (rolA, rolB, rolC, and rolD) of TL-DNA have crucial functions in rhizogenesis [4–8]. These four genes correspond to ORF10, ORF11, ORF12 and ORF15 of the eighteen ORFs of TL-DNA, respectively [9].

Sequences similar to TL-DNA have been found in untransformed plants in the genus Nicotiana [10–12]. It was also reported that carrot and morning glory carry sequences similar to T-DNA [13,14]. ORFs of these sequences were named c-rol genes [12]. Ngrol (NgrolB, NgrolC, NgORF13 and NgORF14) and trol (trolC, torf 13-1 and torf 13-2) genes are c-rol genes contained in the genome of Nicotiana glauca and N. tabacum, respectively.
To distinguish the rol genes of TL-DNA from the Ngr genes, we term them Rirol genes. Furner et al. [11] have proposed that c-rol genes were transferred to the progenitor of Nicotiana species from an A. rhizogenes-like ancestor. It has been reported that these ORFs were transcribed in some tissues of plants [12,15–18].

Analysis of independent and synergistic functions of Rirol genes was made by introducing these genes at various combinations into N. tabacum and D. carota [7,19,20]. There are some differences in reaction to these genes between tobacco and carrot. N. tabacum was also used in order to study the function of the Ngr genes [18,21]. However, materials of these studies have DNA sequences similar to that of TL-DNA in their untransformed genome [10–12]. This raised the question of whether the presence of c-rol genes in the genome of N. tabacum and D. carota obscured the function of the transformed Rirol and Ngr genes. We could not deny that the function of rol transgenes might be suppressed in plants by gene silencing or other mechanisms. Thus, we became involved with the analysis of the function of Rirol and Ngr genes in plants that do not have c-rol genes in their genomes. We attempted root induction with several species of plants and chose a species of the genus Nicotiana, N. debneyi, as the material in this report. N. debneyi belongs to a subgenus Petunioideae that does not include N. glauca and N. tabacum [22]. Southern blot analysis has revealed that the genome of N. debneyi does not contain the c-rol genes [11]. Herein, we investigated the function of root induction by the Rirol and Ngr genes on leaf segments of N. debneyi. The differences in function between the genes of plants and bacteria are analyzed and transition of the function of Ngr genes during the evolution of N. glauca was discussed.

2. Materials and methods

2.1. Cultivation of plants

Seeds of N. debneyi were obtained from Japan Tobacco Inc. They were sterilized for 10 min in a 1.5% solution of sodium hypochlorite. The seeds were germinated aseptically on 35 ml of Murashige and Skoog's medium (MS medium; [23]), that had been solidified with 0.2% (weight/volume) Gellan gum in 100-ml Erlenmeyer flasks. Seedlings were grown in a controlled environment room at \( \approx 25^\circ C \) under continuous light.

2.2. Construction of plasmids

Ngr genes were subcloned from clone \( \lambda N_{31} \) [10], which included the TL-DNA-similar region from the genome of N. glauca. Rirol genes were subcloned from plasmid pLJ1, which contains the TL-DNA region of pRiHRI of A. rhizogenes strain HRI [2]. The DNA fragment of 7718 bp containing the NgrB, NgrC, NgORF13 and NgORF14 loci of \( \lambda N_{31} \) (Fig. 1) was excised with SpeI and XhoI. Plasmid pNBC1314 was obtained by subcloning this fragment into the T-DNA region of the binary vector pMM454-Km (kindly provided by Dr M. Sekine, Nara Institute of Science and Technology, Japan). pMM454-Km has a kanamycin resistance gene (neomycin phosphotransferase) and a lacZ gene (\( \beta \)-galactosidase) with cloning sites in its T-DNA region (Sekine M., unpublished). Similarly, pNB was constructed by

---

**Fig. 1.** Schematic representation of several constructs and comparison of similar regions of A. rhizogenes and N. glauca. The top solid bars represent the map of ORFs in the TL-DNA of pRiA4b of A. rhizogenes. The bottom open bars represent the map of Ngr genes in the genome of N. glauca. Bars above the lines show transcripts transcribed from left to right and those below the lines indicate transcripts transcribed from right to left [9,11,15]. The differing constructs are indicated by solid or open bars between the maps. Restriction endonucleases used in this study are also printed in the map and at the sides of DNA fragments. These fragments were subcloned into the binary vector pMM454-Km. Sp, SpeI. X, XhoI. Sa, Sall. Sm, SmaI. P, PvuI. LB, left border of TL-DNA. RB, right border of TL-DNA.
subcloning the SpeI-SalI fragment of 2972 bp into pMM454-Km. Construction of pRBC1314 and pRB (shown in Fig. 1) was described previously [20].

2.3. Inoculations of leaf segments to induce hairy roots

_A. tumefaciens_ EHA101, that harbored constructs derived from the binary vector pMM454-Km, was tested for its ability to induce the formation of roots on leaf segments of _N. debneyi_. Inoculation was performed as described previously [20]. The leaves were removed from _N. debneyi_ plants 2 or 3 months after sowing. A segment near the basal side of each leaf was cut transversely to ≈2 cm in length. These leaf segments were inserted in the solid MS medium; care was taken to randomize the distribution of segments from different plants and to place each segment with its apical end downwards. Six leaf segments were cultured per flask. Leaf segments were incubated for 3 days and then inoculated with _Agrobacterium_. A wire loop was used to apply a small amount of _A. tumefaciens_. After the inoculation (3–5 days), segments were transferred to MS solid medium supplemented with carbenicillin (500 mg/l) and scored daily for root induction for 30 days.

3. Results

3.1. Adventitious roots on the leaf segments of _N. debneyi_

Adventitious roots were induced from the leaf segments of _N. debneyi_. For the control of the following infection experiments with rol genes, _A. tumefaciens_ strain EHA101 harboring the binary vector pMM454-Km was introduced to leaf segments of _N. debneyi_. pMM454-Km did not have any rol homologues in its T-DNA region. Infection was carried out on the basal side of cut surfaces of leaf segments. About 5 days after the inoculation, all the wound edges of the leaf segments developed pale green callus on the cut midribs, whether or not these leaves were infected with _A. tumefaciens_ and whether or not they induced roots. About a week after the infection, adventitious roots protruded from a few leaf segments, as shown in Fig. 2A. We could hardly observe the induction of roots on the leaf tissues apart from the wounded veins. The frequency of root production was analyzed by using many leaf segments (48 leaf segments of 2-month-old plants and 78 leaf segments of 3-month-old plants). When leaves of 2-month-old plants were used, more than half of the leaf segments induced roots at 30 days after the infection (Fig. 3A). In contrast, only ten out of 78 leaf segments of 3-month-old plants produced adventitious roots (Fig. 3B). The younger leaf segments may have more potential for adventitious root induction than the older ones.

3.2. Induction of hairy roots by wild-type Ri plasmid in _N. debneyi_

_A. tumefaciens_ strain R1000 (pRiA4b), which had been cured of the Ti plasmid and transformed with pRiA4b, a wild-type Ri plasmid (kindly provided by Dr H. Kamada, Tsukuba University, Japan), was introduced into the leaves of _N. debneyi_. Inoculated leaf segments induced many adventitious roots from their cut surfaces, as shown in Fig. 2B. Roots grew rapidly and plagiotropically. These phenotypes are the characteristic features of hairy roots [24]. Leaf segments infected by either of the two plasmids, pRiA4b or pMM454-Km, began to induce adventitious roots about a week after the inoculation (Fig. 3A,B). The beginning of adventitious root production did not seem to be accelerated by the infection with _A. tumefaciens_ harboring the wild type Ri plasmid. Once the root induction began, however, leaf segments inoculated with pRiA4b produced adventitious roots vigorously (Fig. 3A,B). Adventitious roots emerged from all the 126 leaf segments a month after inoculation. The older leaves induced as many roots from the infected leaves as the younger leaves (Fig. 3B). These results indicate that pRiA4b can strongly exhibit its rooting function in _N. debneyi_.

3.3. Function of Rirol genes in _N. debneyi_

It has been reported previously that the RirolB gene included the key for the hairy root induction in _N. tabacum_ [4,7,20]. However, the single gene RirolB was not sufficient for the induction of roots in _D. carota_ [19]. Then, the function of this gene was examined in _N. debneyi_. A DNA fragment
including RirolB of TL-DNA of pRiHRI was subcloned into the binary vector pMM454-Km (Fig. 1; [20]). When the resulting plasmid (pRB) in *A. tumefaciens* strain EHA101 was introduced to leaves of *N. debneyi*, adventitious roots appeared from the cut midribs of many leaves (Fig. 2C). These pRB roots did not exhibit apparent features of hairy root syndrome. The frequency of rooting by pRB was, however, significantly greater than that by the control vector (Fig. 3A,B).

Previous reports have shown that the Rirol genes, RiORF13 and RiORF14 have synergistic function in hairy root induction in *N. tabacum* and *D. carota* [7,8,19–21]. To investigate the synergistic function of these genes in the plant that does not contain a rol-similar sequence, pRBC1314 that contains the RirolB, RirolC, RiORF13 and RiORF14 in the T-DNA region of a binary vector was introduced to *N. debneyi* (Fig. 1). Many adventitious roots were produced, as shown in Fig. 2(D). The frequency of rooting and feature of adventitious roots from leaves induced by pRBC1314 was compared with those by pRB and pRiA4b. Infection with pRBC1314 induced roots on more leaves than that with pRB (Fig. 3A,B). This plasmid induced a similar number of roots on leaf segments of *N. debneyi* as pRiA4b. The region including RirolC, RiORF13 and RiORF14 may enhance the root-inducing function of the RirolB gene in *N. debneyi*. These pRBC1314 roots grew rapidly and showed the reduced geotropic character (Fig. 2D). It was observed that the properties of hairy roots were better achieved through introduction with pRBC1314 than pRB.

**Fig. 2.** Phenotypes of adventitious roots induced by some constructs on *N. debneyi* leaves (bar 1 cm). (A) Control vector (pMM454-Km). (B) Wild-type Ri plasmid (pRiA4b). (C) pRB. (D) pRBC1314. (E) pNBC1314. (F) pNB.
3.4. Function of Ngrol genes in N. debneyi

The function of the Ngrol genes was then analyzed. pNBC1314, which included NgrolB, NgrolC, NgORF13 and NgORF14 in pMM454-Km, was prepared and introduced into N. debneyi leaves (Fig. 1). These genes correspond to the examined four Rirol genes. As shown in Fig. 3(A,B) pNBC1314 could not induce any more adventitious roots than the control vector. These pNBC1314 roots did not exhibit any abnormality of the phenotypes of hairy roots (Fig. 2E). The Ngrol genes may not have any root inducing function with respect to the leaves of N. debneyi.

In order to compare the function between the NgrolB and RirolB genes in N. debneyi, pNB that contained the NgrolB gene in pMM454-Km were prepared. Infection with A. tumefaciens strain EHA101 harboring pNB did not actively provoke adventitious root induction on N. debneyi leaves. The roots on the leaves infected with bacteria harboring pNB did not show any traits of hairy root syndrome (Fig. 2F). The frequency of rooting with pNB did not exceed that with the control vector (Fig. 3A,B). These results indicate that the NgrolB sequence does not contain the same information as the RirolB sequence, for root induction.

4. Discussion

We studied the adventitious root induction of rol homologues on N. debneyi leaves in order to study the function of these genes in a plant that does not have c-rol genes in its genome. When Agrobacterium harboring the wild-type Ri plasmid was infected, leaf segments of N. debneyi induced hairy roots vigorously. Although the single gene RirolB induced adventitious roots, introduction of pRBC1314 resulted in more intense and earlier root formation than that of pRB. More intense traits of hairy root syndrome were also observed with pRBC1314 than with pRB. Therefore, the available data showed that the RirolB gene played a key role in the induction of hairy roots in N. debneyi and the feature of hairy roots is established by the function of RirolC, RiORF13 and RiORF14, suggesting that the activity of these genes is synergistic in this plant. However, the phenotype of the hairy roots induced by pRBC1314 seemed to be slightly less intense than those by pRiA4b. pRiA4b has many genes other than RirolB, RirolC, Ri ORF13 and RiORF14. Some of these genes, such as rolA or the auxin biosynthetic genes, may be required for the full expression of hairy root syndrome [2,7,19,20].

The results of the present study showed that Ngrol genes did not induce adventitious roots in N. debneyi. Infection with A. tumefaciens harboring Ngrol genes did not provoke root induction in tobacco leaf segments [18]. We may, therefore, suppose that the reason for the differences in function between Ngrol and Rirol genes is not

Fig. 3. Root formation on leaf segments of N. debneyi. The number of segments with roots is shown as the percentage of the total number of segments. Leaf disks were inoculated with A. tumefaciens harboring construct pRBC1314 (○), pRB (□), pNBC1314 (●), pNB (■), or pRiA4b (△), or with a control vector (▲). (A) Root induction in leaves taken from N. debneyi plants 2 months after sowing. Forty-eight leaf segments in eight flasks were used for each construct. Bars indicate S.E. (n = 8). (B) Root induction in leaves taken 3 months after sowing. Seventy-eight leaf segments in 13 flasks were used. Bars indicate S.E. (n = 13).
caused by whether or not the recipient of these genes already had c-rol genes in its genome. Tepfer [24] has reported that transgenic plants regenerated shoots from hairy roots when grown in pots. Seeds of these plants inherited the T-DNA genes in their genome. It has been proposed that the genome of some Nicotiana species has c-rol genes as a result of the ancient infection by an A. rhizogenes-like ancestor [11,12]; such an ancient infection may have induced hairy roots to the progenitor of these species [12,18,25]. The present day Ngrol genes, however, did not induce hairy roots or other phenotypes caused by cell division in leaves of N. tabacum and N. debneyi. Function of the Ngrol genes of today and ancient genes transferred from the A. rhizogenes-like ancestor might have diverged during the evolution of Nicotiana species. The differences in function between the bacterial and plant genes may be caused by the diversity in the region of the c-rol sequence. It was proposed that nonsense point mutations in the NgrolB gene might have occurred and the function of this gene might have been lost [18]. Some reports have further shown that the NgrolC, NgORF13 and NgORF14 genes can play their roles when these genes are combined with the RirolB gene or overexpressed [18,21]. It was suggested that the functions of NgORF13 and RiORF13 might have slightly diverged after the infection by the A. rhizogenes-like ancestor [21]. Further studies of the Rirol and Ngrol genes in N. debneyi should clarify their physiological function and molecular evolution.

Acknowledgements

We wish to thank Dr M.P. Gordon (University of Washington, USA) for the gift of a clone of cT-DNA, Dr L. Jouanin and Dr D. Tepfer (INRA, France) for their gift of the cosmid clone, pLJ1. We are also grateful to Dr M. Sekine (Nara Institute of Science and Technology, Japan) and Dr H. Kamada (University of Tsukuba, Japan) for providing the plasmid pMM454-Km and A. tumefaciens R1000 (pRiA4b), respectively. We thank Dr H. Adachi (University of Tokyo), Dr M. Edamatsu (University of Tokyo) and Dr M. Ito (Chiba University, Japan) for many helpful discussions. This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, a Grant-in-Aid for Scientific Research to K.S. from the Ministry of Education, Science, Sports and Culture, Japan and a grant to K.S. from Japan Tobacco Inc.

References


